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(71) Applicant *(for all designated States except US)*: **DEUTSCHES KREBSFORSCHUNGSZENTRUM STIFTUNG DES ÖFFENTLICHEN RECHTS** [DE/DE]; Im Neuenheimer Feld 280, 69120 Heidelberg (DE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **JEDLITSCHKY, Gabriele** [DE/DE]; Friedenstrasse 7/1, 69121 Heidelberg (DE). **KEPPLER, Dietrich** [DE/DE]; Im Hassel 41 A, 69221 Dossenheim (DE).

(74) Agent: **SCHÜSSLER, Andrea**; Huber & Schüssler, Truderingerstrasse 246, 81825 München (DE).

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(54) Title: USE OF INHIBITORS TO MULTIDRUG RESISTANCE PROTEIN 5 (MRP5) TO ENHANCE INTRACELLULAR LEVELS OF CYCLIC NUCLEOTIDES

(57) Abstract: The present invention relates to a novel method for enhancing intracellular levels of cyclic nucleotides, in particular cyclic guanosine monophosphate (cGMP). The method involves administering to a host a therapeutically effective amount of an inhibitor to both MRP5 and phosphodiesterase or administering an inhibitor to MRP5 in combination with an inhibitor to phosphodiesterases. Also, methods for the identification of MRP5 inhibitors, for treating conditions under which higher intracellular levels of cGMP are desirable or for treating MRP5-mediated drug resistance are provided.

USE OF INHIBITORS TO MULTIDRUG RESISTANCE PROTEIN 5 (MRP5) TO ENHANCE INTRACELLULAR LEVELS OF CYCLIC NUCLEOTIDES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention hinges on the surprising discovery that the multidrug resistance protein isoform 5 (MRP5 gene symbol *ABCC5*) transports cyclic nucleotides from cells and that this transport function can be inhibited, thus, enhancing intracellular levels of cyclic nucleotides, such as cyclic 3',5'-guanosine monophosphate (cGMP). Because of the myriad cellular pathways that may be modulated by cGMP, the subject methods have numerous applications in the fields of bronchial and coronary medicine, anti-inflammatory responses, and chemotherapy of tumors and resistant virus-infected cells.

More specifically, the present invention relates to the use of MRP5 inhibitors to treat or prevent pathophysiological conditions or diseases. Especially, the invention relates to a method for enhancing the intracellular levels of cyclic nucleotides, in particular cGMP, to treat or prevent conditions

or diseases such as bronchial asthma, coronary disease, angina pectoris arterial hypertension, erectile dysfunction, or other conditions or diseases where the relaxation of smooth muscle and an enhanced cGMP level in other cell types is desired.

2. Description of the Related Art

Lipid membranes are virtually impermeable to cyclic nucleotides, but the appearance of these intracellular signal molecules (or second messengers) in blood and urine has been known for decades (Butcher et al., *J. Biol. Chem.* 237: 1244-50 (1962); Ashman et al., *Biochem. Biophys. Res. Commun.* 11: 330-4 (1963) (1995)).

Cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger that regulates intracellular reactions in prokaryotes and animal cells. Some hormone-induced cellular responses mediated by cAMP include thyroid hormone synthesis and secretion, cortisol secretion, progesterone secretion, glycogen breakdown, bone resorption, increase in heart rate and force of contraction, water resorption, and triglyceride breakdown (Albersts et al., *Molecular Biology of The Cell*, 2nd Ed., Garland Publishing, Inc., New York, 1989). In many cell types, formation of the cyclic nucleotide 3',5-cAMP has been shown to be accompanied by its secretion from these cells (reviewed in

Brunton et al., *Methods Enzymol.* 159: 83-93 (1988), Fehr et al., *J. Biol. Chem.* 265: 10974-80 (1990)).

Cyclic guanosine monophosphate (cGMP) is another cyclic nucleotide that participates in cell signaling. cGMP is known to activate G-kinase which phophorylates target proteins. Further, export of 3',5-cGMP, upon stimulation of guanylyl cyclases in response to nitric oxide or natriuretic peptides, has been demonstrated in many cells, including endothelial cells, vascular smooth muscle cells, kidney epithelial cells, and lung fibroblasts (Hamet et al., *J. Biol. Chem.* 264: 12364-9 (1989); Woods et al., *Biochem. Pharmacol.* 41: 385-94 (1991); Billiar et al., *Am. J. Physiol.* 262: C1077-82 (1992); Patel et al. *J. Pharmacol. Exp. Ther.* 273: 16-25 (1995)).

In addition, a role of extracellular cyclic nucleotides in cell-cell cross-talks has been suggested (Patel et al. *J. Pharmacol. Exp. Ther.* 273: 16-25 (1995); Friedlander et al., *Kidney Int.* 47: 1500-6 (1995); Millul et al., *Am. J. Physiol.* 39: C1051-60 (1996)). For example, extracellular cAMP acts as a chemoattractant and differentiation factor in the slime mold *Dictyostelium discoideum* by binding to surface receptors for cAMP (Janssens et al., *Microbiol. Rev.* 51: 396-418 (1987)).

As stated above, secretion or export has been considered as a means of removing excessive amounts of intracellular cyclic nucleotides. A second

means of removing excess intracellular cyclic nucleotides involves phosphodiesterases which hydrolyze the cyclic nucleotide to its 5'-monophosphate form. For example, cyclic AMP phosphodiesterase hydrolyzes cyclic AMP to adenosine 5'-monophosphate (5'AMP). Thus, the cellular elimination pathways for these second messengers comprise metabolic degradation by phosphodiesterases, as well as export across the plasma membrane.

For both cAMP and cGMP, secretion has been shown to be unidirectional and energy-dependent (Fehr et al., *J. Biol. Chem.* 265: 10974-80 (1990); Hamet et al., *J. Biol. Chem.* 264: 12364-9 (1989); Woods et al., *Biochem. Pharmacol.* 41: 385-94 (1991); Billiar et al., *Am. J. Physiol.* 262: C1077-82 (1992); Patel et al. *J. Pharmacol. Exp. Ther.* 273: 16-25 (1995); Millul et al., *Am. J. Physiol.* 39: C1051-60 (1996)). Furthermore, the active transport of cGMP has been demonstrated directly utilizing inside-out membrane vesicles from human erythrocytes (Sager et al., *Scand. J. Clin. Lab. Invest.* 56: 289-93 (1996); Schultz et al., *Biochemistry* 37: 1161-6 (1998); Sundkvist et al., *Biochim. Biophys. Acta* 1463: 121-30 (2000)). The transport of both cyclic nucleotides has been shown to be inhibited by probenecid, suggesting that this export is mediated by a transporter for amphiphilic anions

(Millul et al., *Am. J. Physiol.* 39: C1051-60 (1996); Schultz et al., *Biochemistry* 37: 1161-6 (1998); Podevin et al., *Biochim. Biophys. Acta* 629: 135-42 (1980)).

Members of the multidrug resistance protein (MRP) family have been recognized as export pumps for amphiphilic anions, particularly for conjugates of lipophilic compounds with glutathione or several other anionic residues. The best characterized members with respect to transport function are MRP1 (Cole et al., *Science* 258: 1650-4 (1992)), first identified as conjugate export pump in 1994 (Jedlitschky et al., *Cancer Res.* 54: 4833-6 (1994); Leier et al., *J. Biol. Chem.* 269: 27807-10 (1994); Müller et al., *Proc. Natl. Acad. Sci. U.S.C.* 91: 13033-7 (1994)), and the apically localized MRP2, also termed canalicular multidrug resistance protein, cMRP (Büchler et al., *J. Biol. Chem.* 271: 15091-8 (1996)), or canalicular multispecific organic anion transporter, cMOAT (Paulusma et al., *Science* 271: 1126-8 (1996), Taniguchi et al., *Cancer Res.* 56: 4124-9 (1996)) (for reviews see Hipfner et al, *Biochim. Biophys, Acta* 1461: 359-76 (1999) and König et al., *Biochim. Biophys. Acta* 1461: 377-94 (1999)). The identification of MRP3, MRP4, and MRP5 was mainly based on expressed sequence tag data base analyses (Allikmets et al., *Hum. Mol. Genet.* 5: 1649-55 (1996)) followed by cloning of cDNA fragments (Kool et al., *Cancer Res.* 57: 3537-47 (1997)).

MRP5 has been shown to be ubiquitously expressed with high transcript levels in brain, skeletal muscle, lung, and heart and only low transcript levels in liver (Kool et al., *Cancer Res.* 57: 3537-47 (1997), Belinsky et al., *J. Natl. Cancer Inst.* 91: 888-9 (1998), McAleer et al., *J. Biol. Chem.* 274: 23541-8 (1999)). A full-length *MRP5* cDNA has been cloned by several groups (Belinsky et al., *J. Natl. Cancer Inst.* 91: 888-9 (1998), McAleer et al., *J. Biol. Chem.* 274: 23541-8 (1999), Wijnholds et al., *Proc. Am. Assoc. Cancer Res.* 40: 315 (abstract) (1999), Suzuki et al., *Gene* 242: 167-73 (2000)), however, nothing has been reported regarding the physiological function of MRP5 or physiological substrates.

In a recent study, a *MRP5* cDNA, which was cloned from human brain, was expressed in V79 hamster lung fibroblasts. The results identified cyclic nucleotides as physiological substrates for MRP5. Further, these finding suggest that MRP5 transports these cyclic nucleotides from cells (Jedlitschky et al., *J. Biol. Chem.*, 275(39):30069-74, 2000). Therefore, MRP5 may be a pharmacological target for the enhancement of tissue levels of cyclic nucleotides such as cGMP.

Enhancing the intracellular levels of cyclic nucleotides in a host can play a significant role in treating or preventing diseases or conditions associated with low or reduced intracellular levels of cyclic nucleotides. Accordingly,

there is a need in the art for methods to enhance the intracellular levels of cyclic nucleotides. There is also a need in the art for methods to treat and/or prevent diseases or conditions associated with low or reduced intracellular levels of cyclic nucleotides.

SUMMARY OF THE INVENTION

Cellular export of cyclic nucleotides has been observed in various tissues and may represent an elimination pathway for these signaling molecules, in addition to degradation by phosphodiesterases. In a recent study, evidence has been provided that this export is mediated by the multidrug resistance protein isoform MRP5 (gene symbol ABCC5). The transport function of MRP5 was studied in V79 hamster lung fibroblasts transfected with a human MRP5 cDNA. An MRP5-specific antibody detected an overexpression of the glycoprotein of 185 ± 15 kDa in membranes from MRP5-transfected cells and a low basal expression of hamster Mrp5 in control membranes. ATP-dependent transport of 3′,5′-cyclic GMP at a substrate concentration of 1 μM was 4-fold higher in membrane vesicles from MRP5-transfected cells than in control membranes. This transport was saturable with a K_m value of 2.1 μM. MRP5-mediated transport was also detected for 3′,5′-cyclic AMP at a lower affinity, with a K_m value of 379 μM. A potent inhibition

of MRP5-mediated transport was observed by several compounds, known as phosphodiesterase modulators, including trequinsin, with a K_i of 240 nM, and sildenafil, with a K_i value of 267 nM. Thus, cyclic nucleotides are physiological substrates for MRP5; moreover, MRP5 represents a novel pharmacological target for the enhancement of tissue levels of cyclic nucleotides such as cGMP.

Therefore, it is an object of the invention to provide a method for enhancing intracellular levels of cyclic nucleotides.

It is a more specific object of the invention to provide a method for enhancing intracellular levels of cyclic GMP.

It is another object of the invention to provide a method for treating and/or preventing conditions or diseases associated with decreased levels of cyclic nucleotides by administering a therapeutically effective amount of an inhibitor to both MRP5 and phosphodiesterase or administering an inhibitor to MRP5 in combination with an inhibitor to phosphodiesterases.

Many drugs or chemical compounds can be tested to determine if they inhibit MRP5, in addition to phosphodiesterase. Such a test would be beneficial to aid in improving tissue specificity of these drugs.

Therefore, it is another object of the invention to provide a method to test drugs or compounds to improve tissue specificity. The method comprises

comparing the tissue specific expression of MRP5 and phosphodiesterases and the relative potency of inhibition of MRP5 and phosphodiesterases by the drug.

It has been shown that the over-expression of MRP5 in cells can lead to resistance of tumor cells to some cytostatic drugs (Wijnholds et al., *Proc. Natl. Acad. Sci. U.S.A.*, 97: 7476-81 (2000). Specifically, chemotherapeutic drugs such as anti-viral and anti-tumor drugs are rapidly transported out of cells rendering treatment ineffective.

Therefore, it is yet another object of the invention to provide a method for reversing or preventing multidrug resistance mediated by MRP5 or triggering apoptosis in tumor cells comprising administering to a host a therapeutically effective amount of a compound found to inhibit transport function of MRP5 by the method described above.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1:

Expression of MRP5 in transfected V79 cells and human erythrocytes. (A) Northern blot analysis in V79 cells transfected with human MRP5 cDNA (V79-MRP5) an in vector-transfected (V79-Co) or parental (V79) control cells. Total RNA (30 µg) was hybridized under high stringency conditions (Hybridization 12h at 65°c, high stringency wash as described in Jedlitschky et al., *Biochem.*

J., 390: 837-43 (1999)) with a ^{32}P-labeled probe specific for human MRP5 or a β-actin control probe. (B) Immunoblot analysis of MRP5 in crude membrane fractions (100,000 x g Pellets) and purified plasma membrane vesicles (Membranes) prepared from V79-MRP5 and control cells (15 µg of protein per lane). The blot was immuostained using the AMF antiserum detecting both the recombinant human MRP5, as well as endogenous hamster MRP5. (C) MRP5 detection in isolated membranes (30 µg of protein per lane) prepared from transfected V79 cells (including 2 different V79-MRP5 membrane preparations), as well as in membranes from human erythrocytes.

Figure 2:

Transport of cyclic nucleotides into membrane vesicles from MRP5-transfected (V79-MRP5) and vector-transfected control (V79-Co) cells. Membrane vesicles (50 µg of protein) were incubated with [^{3}H]]cGMP (1 µM, upper panels) or [^{3}H]]cAMP (1 µM, lower panels) in the presence of 4 mM ATP (▲) or 4 mM 5'-AMP (■) (left panels), and the vesicle-associated radioactivity was determined as described under Example V. The rates of net ATP-dependent transport (right panels) were calculated by subtracting transport in the presence of 5'-AMP as a blank from transport in the presence of ATP. Under these conditions, ATP-dependent cAMP transport in control membranes was below the detection limit (not shown). ATP-dependent transport of both cyclic

nucleotides in V79-MRP5 membranes is shown in one plot in the lower right panel. Data represent mean values ± S.D. from at least three determinations.

Figure 3:

Kinetic analysis of MRP5-mediated cAMP and cGMP transport and its inhibition by trequinsin. Rates of ATP-dependent transport of [^{3}H]cGMP (upper panel) and [^{3}H]cAMP (lower panel) were determined in V79-MRP5 membranes at five different substrate concentrations as described under Example V. The substrate concentration at half-maximal velocity of transport (K_m values) were calculated from double-reciprocal Lineweaver-Burk plots. In addition, transport of cGMP was analyzed in the presence of 1 µM trequinsin (▲, upper panel), and the inhibition constant (K_i) for competitive inhibition was determined. Points represent mean values ± S.D. from triplicate determinations.

Figure 4:

Inhibitors of MRP5-mediated transport and comparison of their structure with that of cAMP and cGMP.

Figure 5:

Proposed scheme of the regulation of intracellular cGMP levels. The cellular accumulation of cGMP in response to nitric oxide (NO) is determined by a balance between the activities of soluble guanylyl cyclases (sGC), which

catalyze the formation of cGMP from GTP and the elimination of cGMP by metabolic degradation by 3',5'-cyclic nucleotide phosphodiesterases (PDE), as well as by ATP-dependent export by MRP5. Intracellular cGMP receptors of the downstream transduction pathways include cGMP-dependent protein kinases (PKG) and cGMP-gated ion channels. Compounds like sildenafil, trequinsin, and zaprinast can enhance intracellular cGMP concentrations by a dual action on PDEs and ATP-dependent export. The cGMP pumped into the extracelluar space may function, in addition, in cell-cell cross talk.

DETAILED DESCRIPTION OF THE INVENTION

Prior to describing the preferred embodiments, the following definitions are provided.

Host- A host includes humans, non-human primates, non-human mammals, and ungulates. Especially included are agricultural animals, and domestic animals, such as dogs and cats.

In accordance with one embodiment of the invention, there is provided a method for enhancing the intracellular levels of cyclic nucleotides. The method comprises administering to a host a therapeutically effective amount of a composition comprising an inhibitor to both MRP5 and phosphodiesterase or a

composition comprising an inhibitor to MRP5 in combination with an inhibitor to phosphodiesterases.

In another embodiment of the invention, there is provided a method for treating diseases or conditions associated with low or decreased intracellular levels of cyclic nucleotides, in particular cyclic GMP.

For example, an inhibitor to MRP5 and/or to phosphodiesterase can be used to treat diseases or conditions associated with low or decreased intracellular levels of cyclic nucleotides. Essentially, a therapeutic amount of an inhibitor of the invention is administered to a patient in need of treatment. The dose of the inhibitor to be administered can be determined by methods well known in the art. By interfering with the functions of MRP5 or phophodiesterase protein, the inhibitor will prevent the transport of cyclic nucleotides from cells, thus, allowing for the enhancement of intracellular levels of cyclic nucleotides, in particular cGMP.

Diseases and conditions associated with decreased or low levels of cGMP include but are not limited to pathophysiological conditions, such as bronchial asthma, coronary disease, angina pectoris arterial hypertension, erectile dysfunction, or other conditions or diseases where the relaxation of smooth muscle is desired.

By inhibitor it is meant a compound or protein that reduces or suppresses the function of MRP5 such that molecules are no longer transported from the cell. Inhibitors to MRP5 include but are not limited to fluorescein diacetate, probenecid, sulindac, trequinsin, sildenafil and structural analogs of cGMP. With regard to phosphodiestereases, an inhibitor is a compound or protein that reduces or suppresses the function of phosphodiesterase, such as the hydrolysis of cyclic nucleotides. Inhibitors to phosphodiesterase include but are not limited to sildenafil, zaprinast and trequinsin. Additional inhibitors to phosphodiesterases may be found in, for example, Corbin et al., *J. Biol. Chem.*, 274, 13729-32 (1999).

Preferred inhibitors of the invention are inhibitors that have a dual function to inhibit both MRP5 and phosphodiesterase. Such inhibitor are sildenafil, zaprinast and trequinsin. The skilled artisan will recognize, however, that inhibitors to MRP5 that do not have a dual function can be used in combination with inhibitors to phosphodiesterase to achieve the same effect.

Additional inhibitors to treat diseases or conditions associated with low or decreased intracellular levels of cyclic nucleotides include, but are not limited to, a monoclonal antibody, a mixture of monoclonal antibodies, polyclonal antibodies, a mixture of polyclonal antibodies, or a mixture of monoclonal and polyclonal antibodies. Preferred antibodies include anti-MRP5

or anti-phosphodiesterase antibodies produced, for example, in rabbits, mice, and rats. More preferably, a human anti-MRP5 or anti-phosphodiesterase antibody, a humanized anti-antibody, or an anti-MRP5 or anti-phosphodiesterase antibody produced by any method known in the art can be used.

By "humanized antibody" it is meant an antibody which is less immunogenic in humans. This is achieved by various methods known in the art, for example, one can produce a chimeric humanized antibody by grafting the non-human variable domains which retain antigen binding properties onto a human constant region. Additional methods are disclosed in Morrison *et al.*, *Proc. Natl. Acad. Sci.* 81: 6851-5 (1984); Morrison *et al.*, *Adv. Immunol.* 44: 65-92 (1988); Verhoeyen *et al.*, *Science* 239: 1534-1536 (1988); Padlan, *Molec. Immun.* 28: 489-498 (1991); and Padlan, *Molec. Immun.* 31: 169-217 (1994), all of which are hereby incorporated by reference in their entirety.

In another embodiment of the invention, there is provided a composition comprising an inhibitor to MRP5 and/or an inhibitor to phosphodiesterase in a pharmaceutically acceptable carrier, diluent or vehicle which is compatible with the mode or regimen of administration thereof.

The inhibitor composition of the invention may be administered to a human or other animal in an amount sufficient to produce a therapeutic or

prophylactic effect. Such inhibitor compositions of the invention can be administered to such human or other animal in a conventional dosage form prepared by combining the inhibitors of the invention with a conventional pharmaceutically acceptable carrier, vehicle or diluent according to known techniques. It will be recognized by one of skill in the art that the form and character of the pharmaceutically acceptable carrier, vehicle or diluent is dictated by the amount of active ingredient with which it is to be combined, the route of administration and other well-known variables.

The route of administration of the inhibitor composition of the invention may be oral, parenteral, by inhalation or topical. The term parenteral as used herein includes intravenous, intraperitoneal, intramuscular, subcutaneous, rectal or vaginal administration. Subcutaneous and intramuscular forms of parenteral administration are generally preferred.

The daily parenteral and oral dosage regimens for employing compounds of the invention will generally be in the range of about 0.05 to 100, but preferably about 0.5 to 10, milligrams per kilogram body weight per day.

The inhibitor compositions of the invention may also be administered by inhalation. By "inhalation" is meant intranasal and oral inhalation administration. Appropriate dosage forms for such administration, such as an aerosol formulation or a metered dose inhaler, may be prepared by

conventional techniques. The preferred dosage amount of a compound of the invention to be employed is generally within the range of about 1 to 100, preferably 30 to 50 milligrams per kilogram of body weight.

The inhibitor composition of the invention may also be administered topically. By topical administration is meant non-systemic administration and includes the application of an inhibitor composition of the invention externally to the epidermis, to the buccal cavity, a instillation of such an antibody into the ear, eye and nose, where it does not significantly enter the blood stream. By systemic administration is meant oral, intravenous, intraperitoneal and intramuscular administration. The amount of the inhibitor required for therapeutic or prophylactic effect will, of course, vary with the inhibitor chosen, the nature and severity of the disease or condition being treated and the animal undergoing treatment, and is ultimately at the discretion of the physician. A suitable topical dose of an inhibitor of the invention will generally be within the range of about 1 to 100 milligrams per kilogram body weight daily.

Many drugs or chemical compounds can be tested to determine if they inhibit MRP5 in addition to phosphodiesterase. Such a test would be beneficial to aid in improving potency and tissue specificity of these drugs.

Therefore, in another embodiment of the invention there is provided a method to test drugs or compounds to improve potency and tissue specificity. The method comprises comparing the tissue specific expressions of MRP5 and phosphodiesterases and the relative potency of inhibition of MRP5 and phosphodiesterases by the drug.

It has been shown that the over-expression of MRP5 in cells can lead to resistance of tumor cells to some cytostatic drugs. Specifically, chemotherapeutic drugs such as anti-viral and anti-tumor drugs are rapidly transported out of cells rendering treatment ineffective.

Therefore, in yet another embodiment, there is provided a method for reversing or preventing drug resistance mediated by MRP5 or triggering apoptosis in tumor cells comprising administering to a host a therapeutically effective amount of a compound found to inhibit the transport function of MRP5 by the method described above.

Having described the preferred embodiments of the present invention, one skilled in the art will recognize that modifications can be made to the preferred embodiments without altering the scope of the invention.

The following examples are provided to further describe the invention, however, the scope of the invention is not limited thereby.

EXAMPLE I

Cloning of Human MRP5 and Vector Constructions

A full-length *MRP5* cDNA was cloned from a human brain 5'-STRETCH PLUS cDNA library, prepared from whole cerebral brain of a Caucasian male (CLONTECH). Initially, polymerase chain reaction primers were chosen from the partial *MRP5* sequence published by Kool et al. (Kool et al., *Cancer Res.* 57: 3537-47 (1997); GenBank™/EBI accession number U83661) and used for amplification of a 600-bp fragment from the 3'-end of the *MRP5* sequence. This fragment was used as a probe for screening of this library by plaque hybridization, performed as described (Jedlitschky et al. *Biochem. J.* 340: 837-43 (1999)). The screening yielded a 1.6-kb partial *MRP5* clone. Subsequently, the missing 5'-half of the full-length sequence was obtained by polymerase chain reaction using gene-specific primers based on the sequence published by Belinsky et al. (Belinsky et al., *J. Natl. Cancer Inst.* 91: 888-9 (1998); GenBank™/EBI accession number AF104942). Overlapping polymerase chain reaction fragments were amplified and subcloned into the pGEM®-T Easy vector (Promega). Several clones from each fragment were subcloned and sequenced to identify and eliminate mutations introduced by *Taq* polymerase misreading. The 1.6-kb clone, together with the overlapping polymerase chain reaction products, yielded a 4.5-kb cDNA with an open

reading frame encoding 1437 amino acids. The coding sequence was identical to the published sequence with GenBank™/EBI accession number AF104942, except for the following differences: a 2-bp alteration within a single codon (T,T compared with C,C at nucleotide positions 292 and 293 in the published sequence) results in a conservative substitution of Ala to Val. Another 2-bp alteration within a single codon (nucleotides 1323 and 1325 of the published sequence) results in a substitution of a Ser for a Gly. This difference, however, was also reported by McAleer et al. (McAleer et al., *J. Biol. Chem.* 274: 23541-8 (1999); GenBank™/EBI accession number AF146074) and by Suzuki et al. (Suzuki et al. *Biochem. Biophys. Res. Commun.* 238: 790-334 (1997)) in the truncated MRP5 termed SMRP (GenBank™/EBI accession number AB005659).

The complete 4.5-kb cDNA insert was excised from the pGEM®-T Easy vector and cloned into the *Not*I site of the mammalian expression vector pcDNA3.1/Hygro (Invitrogen). The correct orientation and integrity of the cDNA in the expression vector was assessed by restriction analysis and sequencing of the cloning site.

EXAMPLE II

Stable Expression in Mammalian Cells

Chinese hamster lung fibroblasts V79 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and 100 units/ml penicillin/streptomycin. The cells were transfected with the pcDNA3.1/Hygro-*MRP5* cDNA construct or the vector only using FuGENE 6 transfection reagent (Roche Molecular Biochemicals). After 48 h, the cells were split, and stable transfectants were selected using medium containing 600 µg/ml hygromycin B (Invitrogen). Resistant clones were screened by Northern blot and immunoblot analyses for MRP5 expression. Sodium butyrate (5 mM) was added to the cells 40 h before harvesting to enhance the expression of the recombinant protein (Chen et al., *Proc. Natl. Acad. Sci. U.S.A.* 94: 5798-803 (1997), Cui et al., *Mol. Pharmacol.* 55: 929-37 (1999)).

EXAMPLE III

Northern Blot Analysis

Total RNA (30 µg), isolated from transfected cells using the RNeasy kit (Qiagen), was fractionated on a 1.2% formaldehyde/agarose gel and transferred to Duralon UV membranes (Stratagene). A ^{32}P-labeled 970-bp fragment of the *MRP5* cDNA or a β-actin control probe were used for detection. Membranes

were hybridized and washed with high stringency as described (Jedlitschky et al. *Biochem. J.* 340: 837-43 (1999)).

EXAMPLE IV

Immunoblot Analysis

Membrane fractions were diluted with sample buffer und incubated at 37°C for 20 min prior to separation on a 7.5% SDS polyacrylamide gel. Immunoblotting was performed using a tank blotting system (Bio-Rad) and an enhanced chemfluminescence horseradish peroxidase detection system (NEN Life Science Products).

EXAMPLE V

Vesicle Transport Studies

Plasma membrane vesicles from transfected V79 cells were prepared from hypotonically lysed cells as described (Keppler et al., *Methods Enzymol.* 292: 607-16 (1998)). ATP-dependent transport of ^{3}H-labeled substrates into inside-out membrane vesicles was measured by rapid filtration through nitrocellulose filters essentially as described (Keppler et al., *Methods Enzymol.* 292: 607-16 (1998)). Membrane vesicles (50 µg of protein) were incubated in the presence of 4 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100

µg/ml creatine kinase, and labeled substrate, in an incubation buffer containing 250 mM sucrose, and 10 mM Tris/HCl, pH 7.4. The final incubation volume was 75 µl. The substrate and inhibitor concentrations are given in the respective figure legends. For inhibition studies compounds were added from a stock solution in an appropriate solvent (dimethyl sulfoxide or ethanol, final concentration < 0.5% v/v), and identical concentrations of the vehicle were used in control samples. Aliquots (20 µl) of the incubations were taken at the times indicated, diluted in 1 ml of ice-cold incubation buffer, and filtered immediately through nitrocellulose filters (0.2 µm pore size, pre-soaked in incubation buffer). Filters were rinsed with 5 ml of incubation buffer, dissolved in liquid scintillation fluid, and counted for radioactivity. In control experiments, ATP was replaced by an equal concentration of 5'-AMP. Rates of net ATP-dependent transport were calculated by subtracting values obtained in the presence of 5'-AMP as a blank from those in the presence of ATP. For determination of kinetic constants, transport rates were measured at five different substrate concentrations (0.5-10 µM for cGMP and 20-500 µM for cAMP). K_m values were determined as substrate concentration at half-maximal velocity of transport under these conditions. Similar results were obtained by use of double-reciprocal plots and direct curve fitting to the Michaelis-Menten equation.

EXAMPLE VI

Heterologous Expression of MRP5 in V79 Cells

Human *MRP5* cDNA was cloned from a human brain cDNA library and characterized as described under "Experimental Procedures." A stable clonal cell line (V79-MRP5) was established after transfection of Chinese hamster V79 fibroblasts with the *MRP5*-vector construct and selection in hygromycin B. A control hygromycin-resistant clone was obtained from transfection with the parental vector (V79-Co). Expression of *MRP5* was analyzed first on the mRNA level by Northern blotting (Fig. 1A) performed on total RNA isolated from these cell lines. A transcript of approximately 4.5 kb was detected only in the *MRP5*-transfected cells under high stringency conditions using a 970-bp fragment of the human *MRP5* cDNA as a probe. Further, immunoblot analysis was performed on crude membranes (*100,000 x g Pellets*) and purified plasma membrane vesicles (Membranes) using the polyclonal antibody AMF directed against the carboxyl terminus of MRP5 (Fig. 1, B and C). This antibody specifically detected a diffuse band, characteristic for glycosylated proteins, at 170-200 kDa. This is consistent with the molecular mass of about 220 kDa reported for an enhanced green fluorescent protein-MRP5 fusion protein (McAleer et al., *J. Biol. Chem.* 274: 23541-8 (1999)). A protein with the same apparent molecular mass was detected also in the control cells and was

assumed to represent the endogenous hamster MRP5. The detection signal was, in all V79-MRP6 membrane preparations, about 5- to 8-fold stronger than in the control cells.

In addition, a significant signal was obtained in membranes of human erythrocytes, indicating the expression of MRP5 in red blood cells (Fig. 1C). The antibody showed no cross-reactivity with human MRP1, MRP2, MRP3, or MRP6 (not shown).

EXAMPLE VII

ATP-dependent Transport of Cyclic Nucleotides into Membrane Vesicles from Transfected V79 Cells

ATP-dependent transport of [^{3}H]cGMP and [^{3}H]cAMP, which proceeded into the fraction of inside-out-oriented vesicles, was studied during a 10-min period (Fig. 2). ATP-dependent transport (Fig. 2, *left panels*) was calculated by subtracting the vesicle-associated radioactivity in the presence of 5'-AMP from the values obtained in the presence of ATP. ATP-dependent [^{3}H]cGMP accumulation at the standard concentration of 1 µm was 2.3± 0.6 pmol x mg protein $^{-1}$ at 10 min in vesicles from vector-transfected control cells (V79-Co), which exhibited expression of endogenous MRP5 (Fig. 1, B and C). The membranes from MRP5-transfected cells (V79-MRP5) showed a 4-fold

higher ATP-dependent transport with 9.3 ± 0.9 pmol x mg protein $^{-1}$ at 10 min (Fig. 2, *upper panels*). The amount of [^{3}H]cGMP taken up by the vesicles was markedly decreased by increasing the osmolarity of the extravesicular medium, indicating transport into the intravesicular space. At a concentration of 1 M sucrose (outside the vesicles) the transport rate was 22.2 ± 3.3% (mean ± S.D., $n = 3$) of the value obtained under standard conditions with 250 mM sucrose. The rate of ATP-dependent transport of [^{3}H]cAMP at the same concentration (1 µm) was 2.2 ± 0.4 pmol x mg protein $^{-1}$ at 10 min (Fig. 2, *lower panels*) with V79-MRP5 vesicles. This transport was below the detection limit with membranes from control cells under these conditions and substrate concentration (1 µm). At a concentration of 1 µm, ATP-dependent transport of [^{3}H]cGMP in V79-MRP5 was about 4-fold higher than [3H]cAMP transport (Fig. 2, *lower right panel*). The higher affinity of MRP5 to cGMP is reflected in an apparent K_m of 2.1 ± 0.2 µM for cGMP compared with a K_m value of 379 ± 24 µM for cAMP (Fig. 3). The transport efficiency (V_{max}/K_m) was 2100 µl x mg protein $^{-1}$ x min $^{-1}$ for cGMP and only 90 µl x mg protein $^{-1}$ x min $^{-1}$ for cAMP.

EXAMPLE VIII

Inhibition of MRP5-mediated CGMP Transport

Inhibition studies with several amphiphilic anions are summarized in Table I. ATP-dependent transport of cGMP in V79-MRP5 membranes was inhibited to about 50% by fluorescein diacetate at a concentration of 50 μM. This anionic fluorescent dye had been identified as an MRP5 substrate in accumulation studies in cells transfected with an enhanced green fluorescent protein-MRP5 construct (McAleer et al., *J. Biol. Chem.* 274: 23541-8 (1999)). Probenecid, a commonly used inhibitor of organic anion transporters, inhibited cGMP transport at the same concentration (50 μM) by about 68%. Under the conditions used, inhibition by cAMP was detected only at relatively high concentrations (above 100 μM). This is in line with the relatively high K_m value for this compound (Fig. 3). MK571, a leukotriene receptor antagonist, which inhibits MRP1-mediated transport in sub-micromolar concentrations (Jedlitschky et al., *Cancer Res.* 54: 4833-6 (1994)), had no inhibitory effect on MRP5-mediated transport in concentrations up to 50 μM. Several compounds structurally related to cGMP (Fig. 4) and currently used as phosphodiesterase inhibitors were identified as potent inhibitors of cGMP transport. Trequinsin inhibited MRP5-mediated cGMP transport competitively with a K_i value of 240

nM (Fig. 3). A similarly potent inhibition was observed with sildenafil, with a K_i value of 267 nM (figure not shown).

TABLE I

Inhibition of MRP5-mediated cGMP transport

Compound	Concentration	[^{3}H]cGMP transport
		% of control
None (control)		100
cAMP	500	35.5 ± 6.2
Fluorescein diacetate	50	49.2 ± 6.6
Probenecid	50	31.7 ± 4.0
MK571	50	130.5 ± 25.3
cGMP Analogs/phosphodiesterase inhibitors		
8-Bromo-cGMP	10	69.3 ± 4.2
N^2,2'-O-Dibutyrl-cGMP	10	60.1 ± 4.8
Zaprinast	10	41.0 ± 3.9
	1	65.5 ± 7.9
Trequinsin	1	26.2 ± 4.2
Sildenafil	1	22.0 ± 3.4

Membrane vesicles from V79-MRP5 cells were incubated with [^{3}H]cGMP (1 μM) for 15 min at 37°C in the presence of several amphiphilic anions and compounds known as phosphodiesterase inhibitors at the concentrations indicated. Rates of ATP-dependent [^{3}H]cGMP transport were determined as described in the legend to Fig. 1 and calculated as % of control. The control [^{3}H]cGMP transport in these experiments was 16.2 ± 2.2 pmol x mg protein^{-1} at 15 min. Data represent mean values ± S.D. from three determinations.

EXAMPLE IX

Transport of Glutathione and Glucuronate Conjugates

Because glutathione and glucuronate conjugates are high affinity substrates for MRP1, MRP2, and MRP3 (König et al., *Biochim. Biophys. Acta* 1461: 377-94 (1999)), transport of these compounds was measured in V79-MRP5 and control membranes. No significant MRP5-mediated transport of leukotriene C_4 could be detected in incubations with [^{3}H]leukotriene C_4 in concentrations from 25 nM to 1 μM with various proportions of labeled and unlabeled substrate, yielding final specific activities from 0.2 to 1.7 TBq/mmol. No MRP5-mediated high affinity transport could also be detected with 17β-glucuronosyl [^{3}H]estradiol (0.2-5 μM; 0.2-2.0 TBq/mmol) and [^{3}H]GSSG (1-100 μM; 0.3-3.2 TBq/mmol). Transport of these compounds was well detectable under these conditions in membranes from MRPI-overexpressing cells (not shown).

All references cited herein are hereby incorporated by reference in their entirety.

MATERIALS

[2,8-^{3}H]cAMP (0.8 TBq/mmol) and [8-^{3}H]cGMP (0.3 TBq/ mmol) were obtained from Hartmann Analytic, Braunschweig, Germany. Unlabeled cyclic nucleotides, 8-bromo-cGMP, N^2,2'-O-dibutyryl-cGMP, the phosphodiesterase inhibitors Zaprinast and Trequinsin, and the protein standard mixture (26-180 kDa) for SDS polyacrylamide gel electrophoresis were from Sigma. Sildenafil was extracted from a commercially available 100-mg tablet (Viagra®, Pfizer; obtained from a local pharmacy) and purified by high performance liquid chromatography using a C_{18} Hypersil column as described by Warrington et al. (Warrington et al., *Drug Metab. Dispos.* 28: 392-7 (2000)). MK 571 (3-([{3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl}-{(3-dimethyl-amino-3-oxopropyl)-thio}-methyl]thio) propanoic acid) was obtained from Alexis Corp., San Diego, CA. [14,15,19,20-^{3}H]Leukotriene C_4 (4.2 TBq/mmol), 17β-D-glucuronosyl [6,7-^{3}H]estradiol (1.8 TBq/mmol), and [glycine-2-^{3}H]glutathione ([^{3}H]GSH, 1.6 TBq/mmol) were obtained from NEN Life Science Products. [^{3}H]Glutathione disulfide ([3H]GSSG) was synthesized from [^{3}H]GSH as described (Leier et al., *Biochem. J.* 314: 433-7 (1996)).

Antibodies

The AMP antibody was raised in rabbits against the 14 carboxyl-terminal amino acids of the deduced MRP5 sequence (AM-

FAAAENKVAVKG) coupled to keyhole limpet hemocyanin in a procedure similar to that described previously (Büchler et al., *J. Biol. Chem.* 271: 15091-8 (1996), Schnölzer et al., *Int. J. Pept. Protein Res.* 40: 180-93 (1992)).

DISCUSSION

Cyclic GMP has emerged as a major focus in signal transduction research. Mediating most of the effects of nitric oxide, it plays an important role in smooth muscle relaxation, neutrophil degranulation, inhibition of platelet aggregation, neural communication in the brain, and several other physiological processes (for review see Refs. Moncada et al., *Pharmacol. Rev.* 43: 109-42 (1991) and Gathwaite et al., *Annu. Rev. Physiol.* 57: 683-706 (1995)). On one hand, cellular cGMP levels are determined by the rate of synthesis by guanylyl cyclases and, on the other hand, by the elimination rate. Elimination pathways comprise degradation by phosphodiesterases, as well as active export into the extracellular space (Fig. 5). This is in line with the observation that in cerebral cells and platelets, after stimulation with nitric oxide, the cGMP accumulation is decreased faster than it could be explained solely by the phosphodiesterase activity (Bellamy et al., *Proc. Natl. Acad. Sci. U.S.A.* 97: 2928-33 (2000)). The knowledge about the structure and function of the protein families involved in cGMP synthesis and degradation has grown

vastly the last years, whereas little was known so far about the molecular identity of the proteins that mediate the cellular export. Studies in membrane vesicles from human erythrocytes suggested that cGMP is transported by an organic anion transport ATPase (Sager et al., *Scand. J. Clin. Lab. Invest.* 56: 289-93 (1996), Schultz et al., *Biochemistry* 37: 1161-6 (1998), Sundkvist et al., *Biochim. Biophys. Acta* 1463: 121-30 (2000)). The reported characteristics of this transport system in erythrocytes, including the K_m value of 2.4 µM for cGMP (Schultz et al., *Biochemistry* 37: 1161-6 (1998)) and the lack of affinity for the MRP1 substrate leukotriene C_4 (Sundkvist et al., *Biochim. Biophys. Acta* 1463: 121-30 (2000)), are similar to our findings on MRP5-mediated cGMP transport (see Fig. 3, Table I, and "*Inhibition of MRP5-mediated CGMP Transport*"). There is only some discrepancy with respect to cAMP transport. Based on the observation that 100 µM of cAMP caused only a poor inhibition of cGMP transport, it was concluded that the export system for cGMP was not shared with cAMP. MRP5 mediated transport of [^{3}H]cAMP has been observed, but with a more than 20-fold lower transport efficiency (V_{max}/K_m) than that for cGMP (see Fig. 3 and the second paragraph under 'Results'). Therefore, poor inhibition of cGMP transport by cAMP was obtained at concentrations below 100 µM (Table I). Because of the high K_m value for cAMP (379 µm, Fig. 3), MRP5-mediated cAMP transport may only be

significant under conditions with high intracellular cAMP levels. Because of the reported transport activity, MRP5 expression was analyzed in erythrocyte membranes and a significant signal with the MRP5-specific AMF antiserum was obtained (Fig. 1C). This indicates that MRP5 represents at least a major constituent of the cGMP export in human erythrocytes. Secretion of cGMP in response to nitric oxide has been also observed in rat lung fibroblasts (Patel et al. *J. Pharmacol. Exp. Ther.* 273: 16-25 (1995)). This is in line with observations of a basal cGMP transport in parental or vector-transfected control cells and the detection of a glycoprotein recognized by the MRP5-specific antibody in the control cells (Fig. 1, B and C).

Little was known so far about the function of MRP5. Studies in human embryonic kidney cells transfected with a green fluorescent protein-MRP5 construct indicated that it mediates export of the anionic dye fluorescein diacetate ATP-dependently, but glutathione-independently (McAleer et al., *J. Biol. Chem.* 274: 23541-8 (1999)). This is in agreement with the inhibition of cGMP transport by this compound (Table 1). Unlike MRP1 and MRP2, MRP5 seems not to confer resistance against anthracyclines, Vinca alkaloids, and epipodophyllotoxins (McAleer et al., *J. Biol. Chem.* 274: 23541-8 (1999), Wijnholds et al., *Proc. Am. Assoc. Cancer Res.* 40: 315 (abstract) (1999)). However, MRP5-mediated low level resistance against thiopurines has been

reported (Wijnholds et al., *Proc. Am. Assoc. Cancer Res.* 40: 315 (abstract) (1999)). Comparison of hydropathy profiles of MRP5 with other members of the MRP family indicated that its structure is most similar to that of MRP4. Both lack the hydrophobic extension of about 200 amino acids (first five transmembrane domains) present in MRP1, MRP2, and MRP3 (Belinsky et al., *J. Natl. Cancer Inst.* 91: 888-9 (1998), Borst et al., *Biochim. Biophys. Acta* 1461: 347-57 (1999)). MRP4 has been found to be overexpressed in T-lymphoid CEM-rl cells resistant to nucleoside-based antiviral drugs, and an energy-dependent efflux of the nucleoside phosphonate 9-(2-phosphonyl-methoxyethyl)adenine (PMEA) and of azidothymidine monophosphate from these cells has been demonstrated (Schuetz et al., *Nat. Med.* 5: 1048-51 (1999)). Very recently, an MRP5-mediated efflux of PMEA and 6-thioinosine from transfected human embryonic kidney cells and Madin-Darby canine kidney cells has been reported (Wijnholds et al., *Proc. Natl. Acad. Sci. U.S.A.* 97: 7476-81 (2000)). In these cells an enhanced efflux of S-(2, 4dinitrophenyl)glutathione and of glutathione across the basolateral membrane was observed. In isolated membrane vesicles, we could not detect a significant MRP5 mediated transport of the MRP1 and MRP2 substrates leukotriene C_4, 17β-glucuronosyl estradiol, and glutathione disulfide. In addition, cGMP transport was not inhibited by the cysteinyl leukotriene analog MK571, a potent

inhibitor of MRP1- and MRP2-mediated transport. This, however, does not exclude MRP5-mediated transport of other glutathione and glucuronate conjugates or complexes or transport of these compounds at high substrate concentrations.

The present application identifies for the first time natural cyclic nucleotides as substrates of an ATP-binding cassette transporter of the MRP family. cGMP and cAMP are also the first phosphate substrates for which ATP-dependent transport by an MRP family member was demonstrated directly in isolated membrane vesicles. Other MRP family members, especially MRP4, may function as transporters for cyclic nucleotides, as well. Based on RNase protection assays, however, expression of MRP4 is low in most tissues under normal conditions, whereas MRP5 is abundant in almost every organ, with low expression only in liver (Kool et al., *Cancer Res.* 57: 3537-47 (1997)). Because cGMP is the high affinity substrate for MRP5, this transporter may be a novel pharmacological target to interfere cGMP elimination from cells and enhance the intracellular cGMP concentration under various pathophysiological conditions, especially to relax vascular smooth muscles in treatment of angina pectoris, arterial hypertension, or erectile dysfunction. A relatively potent inhibition of this transport has been demonstrated by several compounds known as phosphodiesterase inhibitors, including sildenafil as the most prominent one.

Thus, these compounds can enhance intracellular cGMP levels through a dual action on cGMP degradation and export (Fig. 5). Compounds, inhibiting more specifically the transport system, may be identified and could be useful to determine the role of the ATP-dependent export in signal termination.

Claims:

1. A method for enhancing intracellular levels of a cyclic nucleotide comprising administering to a host a therapeutically effective amount of an inhibitor to both MRP5 (*ABCC5*) and phosphodiesterase or administering an inhibitor to MRP5 in combination with an inhibitor to phosphodiesterases.

2. The method of claim 1, wherein said cyclic nucleotide is cGMP.

3. A method for treating or preventing conditions or diseases associated with decreased levels of a cyclic nucleotide comprising administering a therapeutically effective amount of an inhibitor to both MRP5 and phosphodiesterases or administering an inhibitor to MRP5 in combination with an inhibitor to phosphodiesterases.

4. A compositions comprising an inhibitor to MRP5 and/or an inhibitor to phosphodiesterases in a pharmaceutically acceptable carrier, diluent or vehicle.

5. A method for the identification of inhibitors, that increase cellular levels of cyclic nucleotides, by use of MRP5-expressing cells or membranes from these cells (screening method).

6. A method to test drugs or compounds to improve tissue specificity comprising comparing the tissue specific expression of MRP5 and phosphodiesterases and the relative potency of inhibition of MRP5 and phosphodiesterases by the drug.

7. A method for reversing or preventing chemotherapy resistance mediated by MRP5 or triggering apoptosis in tumor cells which express MRP5 comprising administering to a host a therapeutically effective amount of an inhibitor to MRP5, identified according to the method of claim 5.

8. The method of claim 7, wherein said inhibitor to MRP5 is a cyclic nucleotide analog.

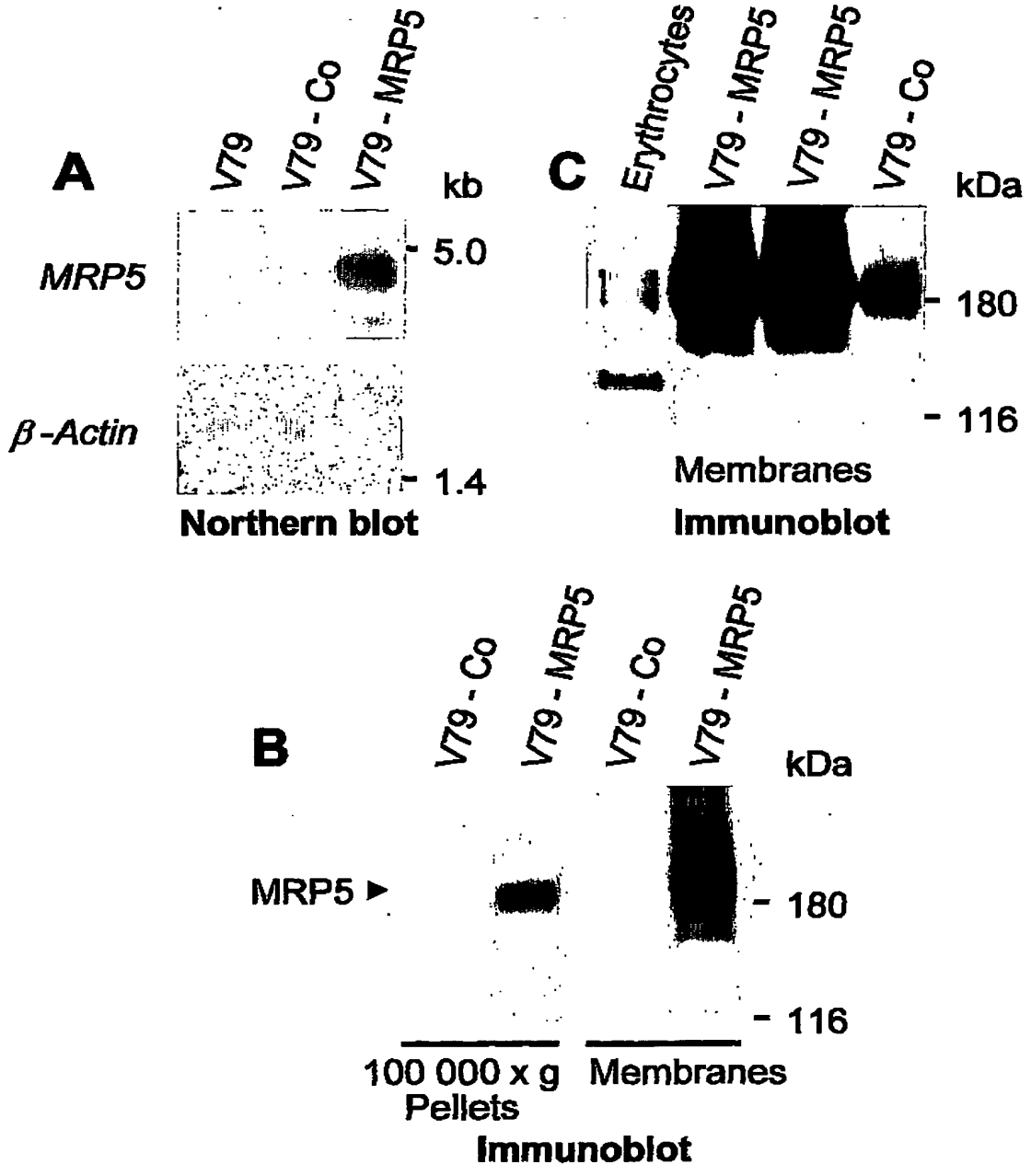

FIGURE 1

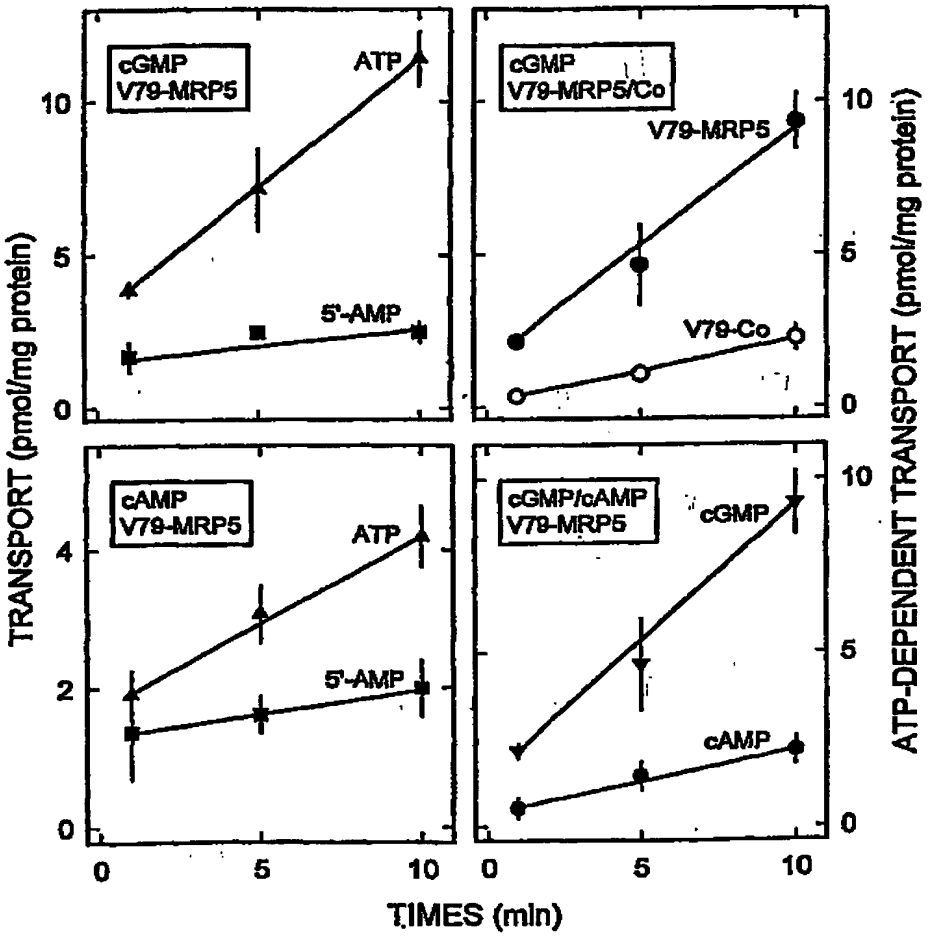

FIGURE 2

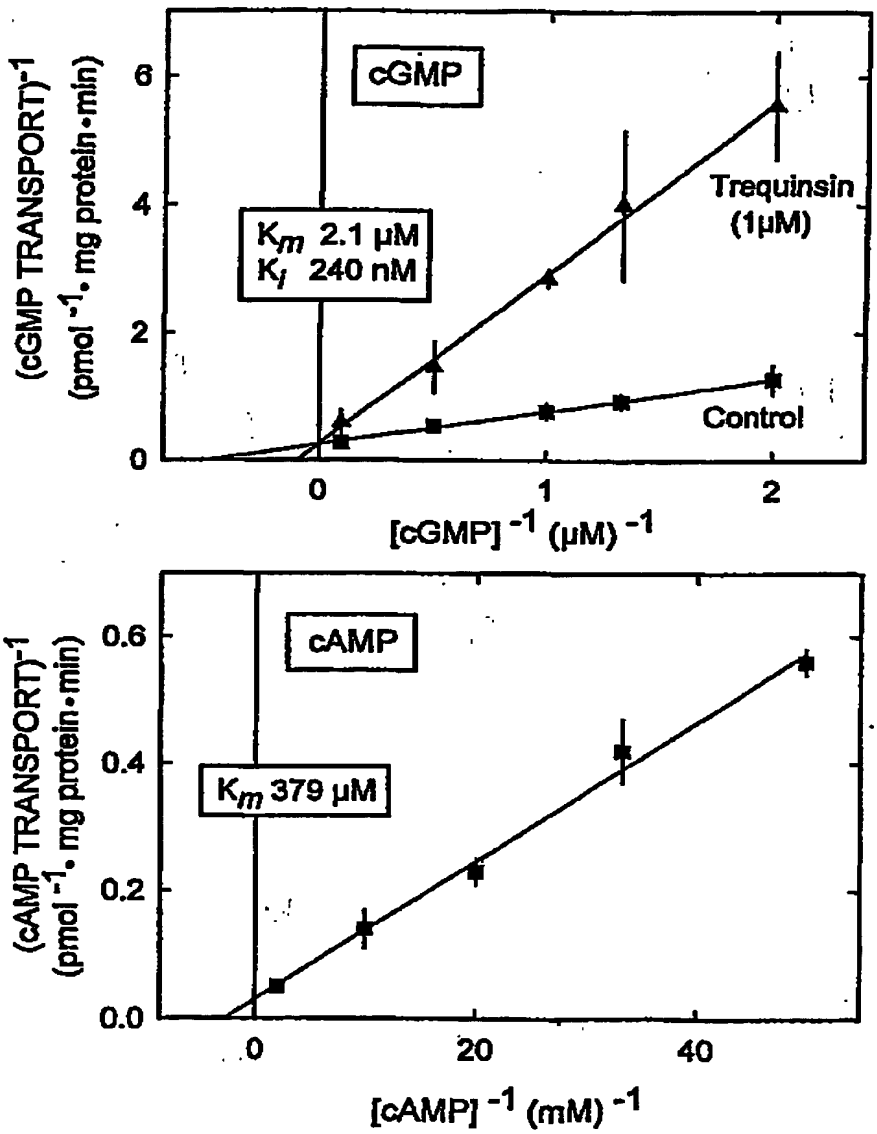
cGMP
K_m 2.1 µM
K_i 240 nM
Trequinsin (1µM)
Control
(cGMP TRANSPORT)$^{-1}$ (pmol^{-1}. mg protein•min)
[cGMP]$^{-1}$ (µM)$^{-1}$
cAMP
K_m 379 µM
(cAMP TRANSPORT)$^{-1}$ (pmol^{-1}. mg protein•min)
[cAMP]$^{-1}$ (mM)$^{-1}$

FIGURE 3

Substrates and Inhibitors of MRP5 (ABCC5)

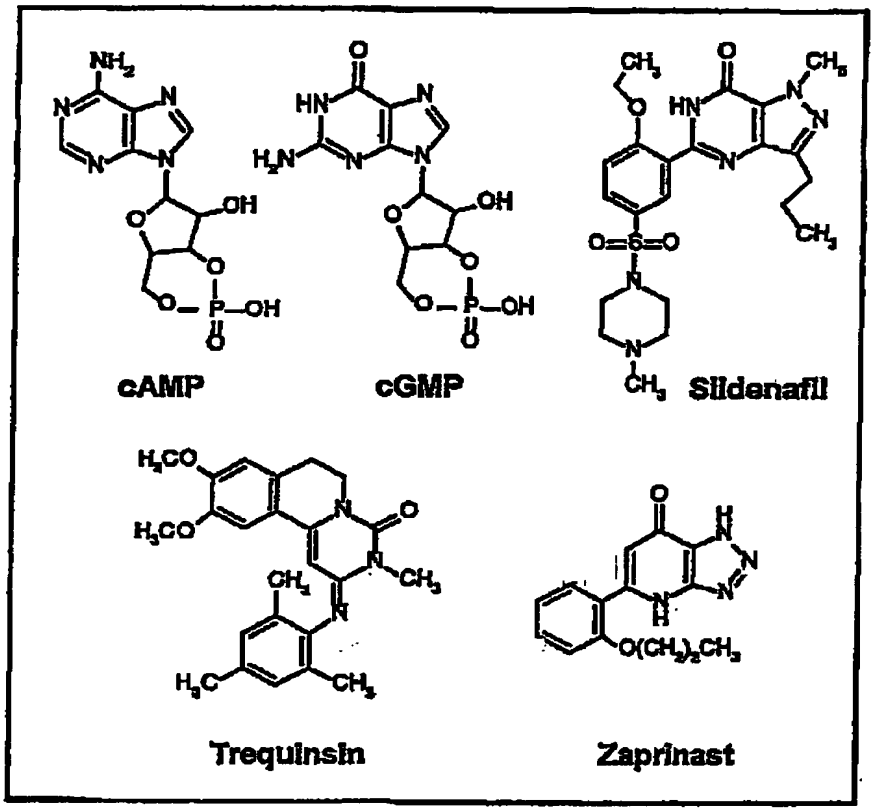

FIGURE 4

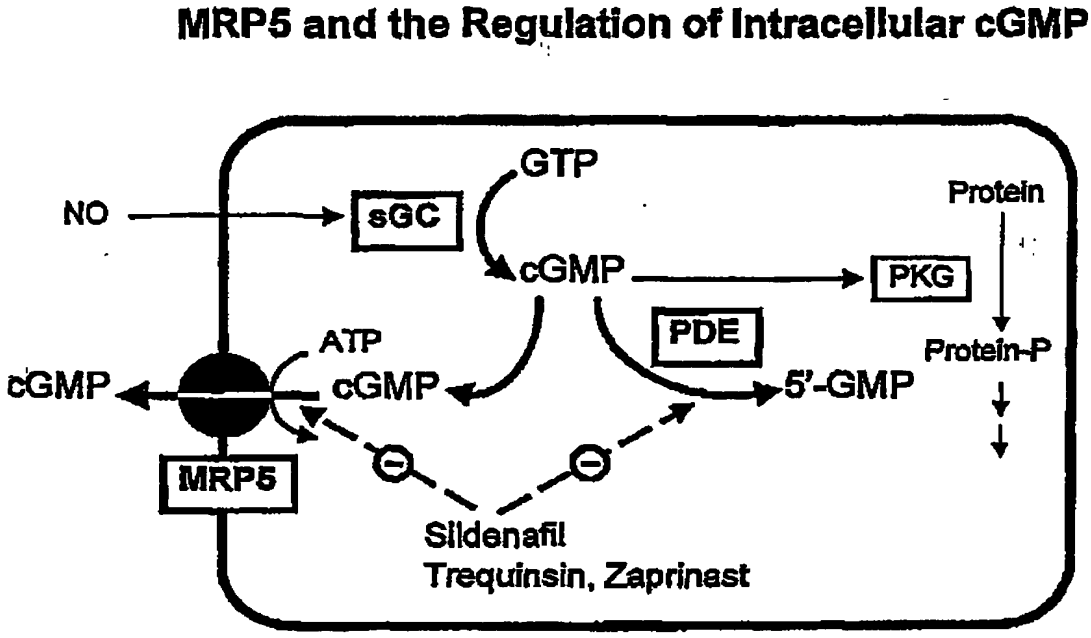
MRP5 and the Regulation of Intracellular cGMP
GTP
NO
sGC
cGMP
PKG
Protein
PDE
ATP
cGMP
cGMP
5'-GMP
Protein-P
MRP5
Sildenafil
Trequinsin, Zaprinast

FIGURE 5